Caffeic Acid Activity Against *Clostridium botulinum* Spores

BOBBY L. BOWLES and ARTHUR J. MILLER

ABSTRACT

Caffeic acid (CA) is widely distributed among higher fruits and vegetables. While CA has antimicrobial activity, little information exists on its utility as a food additive. As such, CA was tested for activity against *Clostridium botulinum* spores. At 0.78 and 3.25 mM, CA inhibited germination for 6 and 24 hr, respectively, with >100 mM required to render spores nonviable. CA concentrations ≥ 50mM reduced 80°C spore thermal resistance. Sporostatic activity was retained when tested in commercial meat broths, and 5.0 mM CA delayed toxigenesis. Caffeic acid has potential as a food additive to inhibit growth of *C. botulinum,* and reduce thermal processing requirements of heat sensitive foods.

Key Words: caffeic acid, antibacterial, microbes, *Clostridium botulinum*, food additives

INTRODUCTION

CLOSTRIDIUM BOTULINUM is an anaerobic, Gram-positive, endosporulating bacillus that synthesizes a potent neurotoxin. The disease induced by the neurotoxin is of international concern with distinct seasonal and strain regional differences (Hauschild, 1989). Foodborne botulism outbreaks are a frequent form of the disease, and are usually associated with home prepared products. Recent U.S. and Canadian (1977-1985) outbreaks were attributed to foods at restaurants (Sugiyama, 1990). Vegetable and fruits are the most often implicated food vehicles, followed by fish and fish products, condiments, and beef or pork products. *C. botulinum* spores are the important initial contaminants, and inhibition is primarily directed against spore transition phases (e.g., activation, germination and/or outgrowth) to ensure botulism-safe foods.

The incidence of botulism is low in the U.S. but toxin potency, health concerns about some antimicrobial agents such as $NaNO_2$, and growth potential in low oxygen-tension packaging, necessitate further investigations to identify alternative antibotulinal agents (Bean and Griffin, 1990; Conner et al., 1989). Organic acids inhibit growth of several bacterial pathogens, and their study as antimicrobial food additives has been reported (Debevere, 1988; Miller et al., 1993; Mountney and O'Malley, 1965; Palumbo and Williams, 1992).

Hydroxycinnamic acids, their methylated and sugar derivatives, are ubiquitous throughout the plant kingdom (Harborne and Conner, 1961). Although hydroxycinnamic acids, such as caffeic acid (CA) are not approved as food additives, they are common naturally occurring components of most daily dietary intakes. The total human dietary intake of plant phenols may approach 1.0 g/day (Brown, 1980). CA reduces the incidence of cancer by preventing *in situ* formation of carcinogens from precursors (Yamaguchi and Iki, 1986). CA and its sugar esters are antibacterial (Ravn et al., 1989; Toda et al., 1989), antifungal (Valle, 1957), antiviral (John and Mukundan, 1979), and inhibit mycotoxin production (Paster et al., 1988). Furthermore, CA interacts with metal ions such as iron (Kontoghioghes et al., 1986), that have been demonstrated to be essential *C. botulinum* growth factors.

Authors Bowles and Miller are with the U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Microbial Food Safety Unit, 600 E. Mermaid Lane, Philadelphia, PA 19118.

The antibotulinal activity of hydroxycinnamic acids is not well defined, and few have been tested for spore inhibition. The inhibitory activity of CA, coumaric, ferulic and sinapic acids against Gram-positive bacteria (Herald and Davidson, 1983; Nowak et al., 1992) suggests, however, that they may have potential as antibotulinal agents. CA is a natural component of raw plant materials for which antibotulinal activity has been reported (Ismaiel and Pierson, 1990). Our objective was to test CA to determine activity against *C. botulinum* spores. The antibotulinal efficacy was defined by assessing its effect on germination rates, dipicolinic acid release, spore thermal resistance, and vegetative cell toxigenesis.

MATERIALS & METHODS

Cultures

A spore mixture containing three type A (33, 62A, 69) and 3 type B (999, 169, ATCC 7949) proteolytic *C. botulinum* strains was used. Individual strain spore suspensions were prepared by culturing in botulinal assay medium (Huhtanen, 1975) without thioglycollate (BAM) for 21 days at 32°C in a flexible anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Anaerobiosis was maintained using a gas mixture consisting of 10% CO_2, 5% H_2, and 85% N_2 by periodic gas-exchange flushes and palladium catalyzed O_2 removal. Spore crops were harvested by 3 successive centrifugations at 17,310 × *g* for 10 min at 5°C with sterile distilled/deionized H_2O washes between centrifugations. Spore pellets were suspended in sterile distilled/deionized H_2O, heat-shocked (10 min at 80°C) and stored at 5°C prior to use. *C. botulinum* confirmation was based on Gram-reaction cellular morphology, neurotoxin production confirmed by mouse bioassay, lipase, catalase, and oxidase activities (Centers for Disease Control, 1974). Each spore crop was quantified and the six-strain spore mixture prepared by combining equal numbers of individual strains to provide a final concentration of 4.7×10^5 spores/mL. Viability and germination rates of individual strains were tested initially, and monthly on spore mixtures (Centers for Disease Control, 1974). All analyses, unless otherwise indicated, were conducted using heat (10 min at 80°C) activated 4.7×10^5 CFU/mL *C. botulinum* spores.

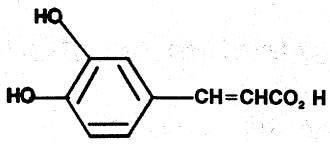
Caffeic acid

CA (cis-butanenedioic acid) was purchased as the 99.9% free acid from Sigma Chemical Company (St. Louis, MO) and used as supplied. A 0.2 M stock solution was prepared in 95% ethanol and stored at 5°C. Final ethanol concentrations of treatments for antimicrobial testing were below those reported to be sporostatic or sporicidal (Koransky et al., 1978). The structure and some properties of CA are listed (Table 1).

Determination of spore minimal inhibitory concentrations (MIC)

A quantitative broth dilution method was used to determine the effect of CA on *C. botulinum* spores (Bowles and Miller, 1993a,b). CA was serially diluted (200, 100, 50, 25, 12.5, 6.25, 3.13...-0.05 mM) in 1.0 mL BAM broth (pH 7.0 ± 0.2), and inoculated. The CA concentrations represented a final pH range 7.0–4.0. The tubes were incubated anaerobically for 6 or 24 hr at 32°C, heat-shocked for 10 min at 80°C to destroy germinated and outgrown spores, and 0.1 mL subcultured into 9.9 mL thioglycollate (TG) broth. A separate set of TG tubes inoculated with non-heat-treated culture was included to determine spore viability without thermal treatment. Five replicate TG tubes inoculated with heat- or nonheat-treated culture, were used for each concentration. TG tubes were incubated aerobically for 48 hr at 32°C, then examined for turbidity. A sporicidal effect was defined as no growth in at least 4 TG tubes containing non-heat-treated cultures. A sporo-

Table 1—Description and properties of caffeic acid

Type	Description[a]
Structure	
Synonyms	3-(3,4-Dihydroxyphenyl)-2-propenoic acid 3,4-dihydroxycinnamic acid
Molecular weight	180.15
Melting point	152–153°C
Decomposition	223–225°C (softens at 194°C)
Normal state (25°C)	Yellow-brown crystals
Food regulatory status	Nonapproved
Carcinogenicity[b]	Gastrointestinal tumors in 6 wk old male rats by 2% oral administration
Natural occurrence	Plants Conjugated form (e.g. chlorogenic acid)
Solubilities	Sparingly in cold H_2O; Freely in hot H_2O or cold alcohol

[a] Budavair et al., (1989).
[b] Hirose et al. (1990).

static effect was defined as growth in at least 4 TG tubes inoculated with heat-treated culture.

Dipicolinic acid (DPA) release

DPA release was estimated using the colorimetric assay of Janssen et al. (1958). CA was added to 9.9 mL BAM broth tubes to provide a concentration series of 0, 1, 2, 3, 4, and 5 mM, with pH range 7.0-5.0. Test media were inoculated and incubated 9 hr anaerobically at 32°C. Thereafter, cultures were centrifuged at 1,500 × *g* for 10 min and the supernatant fluid was sampled for colorimetric analysis. One mL of a freshly prepared 0.5M acetate buffered chromogenic reagent was added to 4.0 mL of the culture supernatant. Optical density was measured at 440 nm using a Shimadzu UV-VIS Model 160 spectrophotometer (Kyoto, Japan) and DPA content calculated from a DPA standard curve (0-160 µg/mL).

Effect of CA on spore thermal resistance

C. botulinum spores (8.2×10^6 CFU/mL) were aerobically exposed to 50 or 100 mM (both pH 4.6) of CA in 5.0 mL glass vials containing BAM broth for 30 min at 25°C, and the exposure medium transferred to an 80°C Exacal high temperature water bath (NesLab Instruments Inc., Newington, N.H.) for 5–20 min. A Keithley Metrabyte datalogger model DDL 4100 (Taunton, MA) was used to monitor temperature and equilibration time. After heat treatment, samples were removed, cooled in an ice bath, and 0.1 mL diluted into 9.9 mL 1% peptone-H_2O (pH 7.2). Diluted samples were plated in duplicate onto BAM agar plates using a Spiral Systems Model D plating instrument (Cincinnati, OH) and incubated anaerobically at 32°C for 48 hr. Plates were enumerated using a Spiral Systems Model 500A, then converted into bacterial counts with Spiral Biotech CASBATM II BEN software (Bethesda, MD). Spore thermal resistance was evaluated by comparing the population densities of CA-treated and -untreated BAM samples. A 50 min exposure control (25°C) of non-heat-treated spores with 100 mM CA was included to confirm spore viability in the absence of thermal treatment.

Inhibitory activity of CA in chicken and beef broth

Commercially prepared canned chicken and beef broths were dispensed in 9.9 mL portions to sterile test tubes and caffeic acid added to provide concentrations of 2.0, 3.0, 4.0 and 5.0 mM, with pH range 7.0–5.0. The broths were inoculated and incubated anaerobically at 25°C and examined visually at 24 hr intervals for turbidity. The commercial chicken and beef broths each contained, (manufacture's description) 1% each (w/v) of protein carbohydrate, and fat. Inhibition was defined by comparing the turbidity of inoculated CA treatments to those of uninoculated and unsupplemented controls.

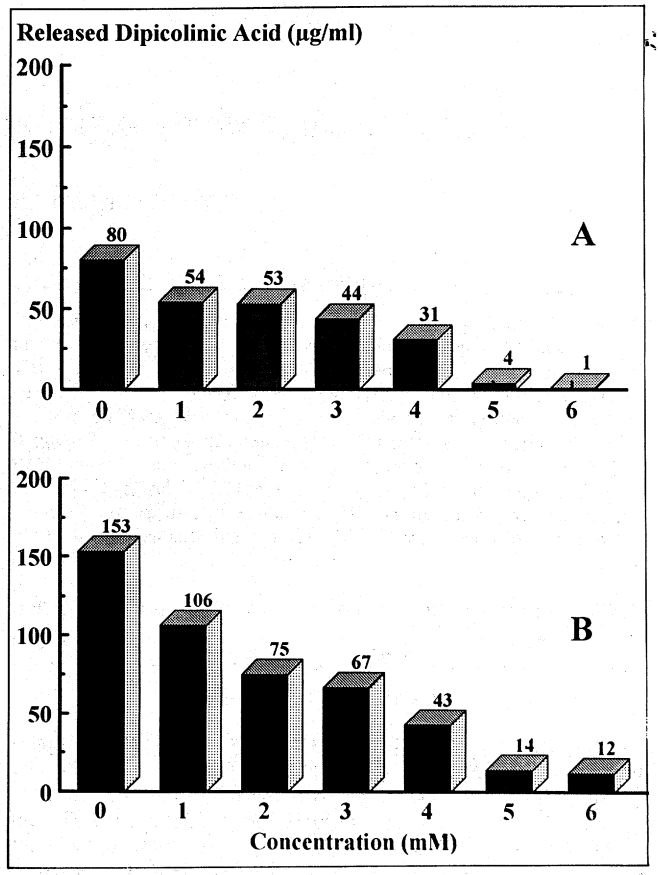


Fig. 1.—Effect of caffeic acid on dipicolinic acid (DPA) release from *C. botulinum* spores in BAM broth after 9 (A) and 24 (B) hr anaerobic incubation at 32°C.

Effect of CA on toxigenesis

Five mL BAM broth tubes containing 0, 5, 10, 25, 50 or 100 mM of CA were inoculated. All tubes, including an uninoculated set of CA test concentration controls, were incubated anaerobically for 48 hr at 32°C, then centrifuged (1500 × *g* for 10 min) to remove cellular debris. A 72 hr bioassay was conducted on duplicate Swiss-Webber 15–20g mice of either sex by intraperitoneal injection (0.5 mL) of undiluted culture supernatant fluid. Polyvalent antiserum controls were included on some samples to confirm clinical symptoms as botulism (Centers for Disease Control, 1974).

RESULTS & DISCUSSION

CA INHIBITED GERMINATION for 6 hr at 0.78 mM and 24 hr at 3.25 mM (data not shown). CA concentrations > 100 mM were sporicidal at both incubation periods (data not shown). Controls with no CA addition germinated, as confirmed by lack of growth in TG broth after heat shocking. Although bacterial spores may remain viable under extreme environmental conditions, germination may be inhibited by minor physical or chemical changes (Gould et al., 1970; Halvorson et al., 1966). Relatively small amounts of a chemical agent can be sporostatic, yet considerably higher concentrations are required to render spores non-viable or to inhibit vegetative cell growth (Bowles, 1991; Bowles and Miller 1993a,b; Smith and Dawes, 1989).

In the presence of CA DPA release was markedly less than unsupplemented controls for up to 24 hr at 32°C. Inhibition was time- and concentration-related, with an average 90% reduction observed at concentrations ≥ 4.0 mM (Fig. 1). Unsupplemented cultures contained 80 and 152.7 µgDPA/mL after 9 and 24 hr at 32°C, respectively. During initial germination phases, degradative processes of cortex embedded lytic en-

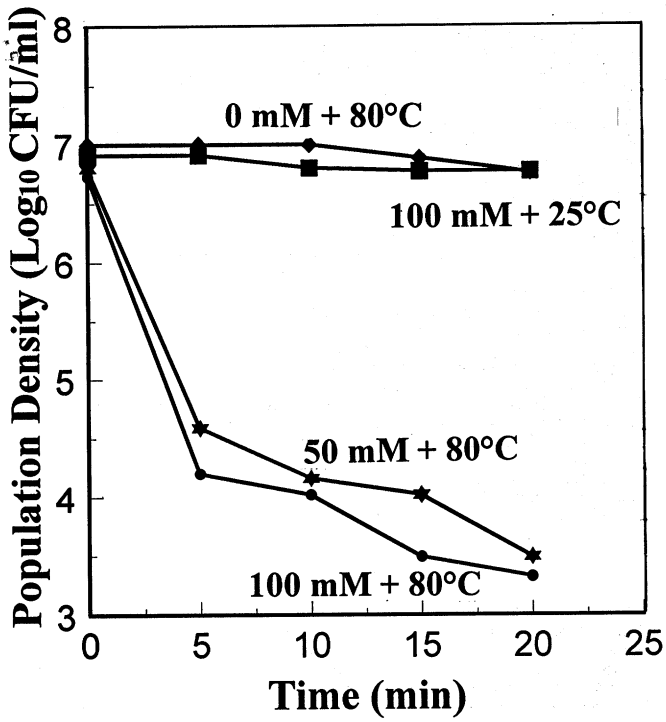


Fig 2.—Effect of caffeic acid on thermal resistance of *C. botulinum* spores in BAM broth. Spores were incubated with various levels of CA at 25°C for 30 min, then transferred to 80°C for 0–20 min. A control was maintained for 50 min at 25°C.

Table 2—Activity of caffeic acid against *C. botulinum* spores in commercial meat broths[a]

Incubation (d)	Minimal inhibitory concentration (mM)	
	Chicken broth	Beef broth
2	2	2
4	2	2
6	3	>5
8	>5	>5

[a] Caffeic acid was tested at 2.0, 3.0, 4.0, and 5.0 mM. Growth was assessed by comparing cultures with unsupplemented (inoculated and uninoculated) commercial broths.

Table 3—Bioassay of the effect of caffeic acid on 48 hr *C. botulinum* challenge in BAM broth at 32°C

Caffeic acid (mM)	Culture toxicity assessment (dead mice/total mice)
0[a]	2/2
C[b]	0/2
5	0/2
10	0/2
25	2/2[c]

[a] Unsupplemented BAM control.
[b] Polyvalent antiserum control, containing spores and no compound.
[c] Mice died of unconfirmed causes.

zymes alter spore permeability with concomitant DPA release (Gould et al., 1970; Halvorson et al., 1966). Reduced DPA release suggested that CA precluded changes in spore coat permeability (Halvorson et al., 1966).

Proteolytic *C. botulinum* spores were reduced by ≤ 100 mM CA for 30 min, followed by 80°C heating for ≤ 20 min (Fig. 2). Temperature equilibrium was reached in 2.7 min and as expected, no decrease in population density occurred in 0 mM CA controls. There was an indirect relationship between the two CA doses and population densities. After 5 min at 80°C a 2.4 or 2.8 $\log_{10}$ CFU/mL reduction occurred at CA levels of 50 and 100 mM, respectively. Inhibition as time-dependent, and after 20 min at 80°C viable spore population densities at 50 and 100 mM CA fell 3.4 and 3.6 $\log_{10}$ CFU/mL with respect to the control. Viability was retained when 100 mM CA was added to challenge spores at 25°C for 50 min, the full chemical exposure time. This indicated that CA had lowered the thermal resistance of proteolytic *C. botulinum* spores to a normally innocuous thermal challenge. Furthermore, although 100 mM samples had a pH of 4.6, there was no evidence that this acid level had any inhibitory effect.

Thermal resistance of sporeforming spoilage organisms has been reduced by edible green plant extracts (LaBaw and Desrosier, 1953), and certain synthetic plant auxins modulated *Bacillus coagulans* spore resistance (LaBaw and Desrosier, 1954). Thermal resistance of *C. botulinum* spores, moreover, may be altered by the Fe^{++} and Ca^{++} levels or fatty acid content in the sporulation substrate (Sugiyama, 1951). CA inhibitions may be attributed to its reactivity at 80°C and changes in the tertiary structure or spore coat proteins. Mild heating activates bacterial spores by inducing spore coat structural changes (Gould et al., 1970; Halvorson, et al., 1966). Thus, spore core incorporation of CA may be enhanced with subsequent structural damage that render spores non-viable. The observed activity suggests CA may be useful to lower thermal processing requirements for some foods.

Two mM CA prevented germination for up to 4 days in both chicken and beef broths, and >5.0 mM was required for inhibition for 8 days at 32°C (Table 2). CA MICs in these foods were ≈5 times higher than those in BAM broth. Antibacterial agents are often less active in foods (Raccach, 1984), because many food components (e.g. protein, fats) can lower activity.

In the mouse bioassay, toxigenesis was delayed or inhibited by CA concentrations ≥ 5.0 mM (Table 3). Unsupplemented controls were toxigenic at 48 hr at 32°C, and the observed clinical symptoms were confirmed as botulism by neutralization with polyvalent antiserum. CA concentrations ≥ 25 mM were lethal. CA has a mouse LD_{50} of > 721 mg/kg (Sigma Chemical Co, St. Louis, MO).

CONCLUSIONS

CAFFEIC ACID, A HYDROXYCINNAMIC ACID DERIVATIVE, is a naturally occurring food component that was demonstrated to be sporostatic and sporicidal, to reduce spore thermal resistance, and to inhibit *C. botulinum* toxigenesis. The antibotulinal properties suggest that CA may have potential to control *C. botulinum* in foods or to lower required thermal processing conditions.

REFERENCES

Bean, N.H. and Griffin, P.M. 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. J. Food Protect. 53: 804–817.

Bowles, B.L. 1991. Studies on the mode of action of phenylglyoxal as an inhibitor of *Clostridium sporogenes*. Ph.D. dissertation, Dept of Biological Sciences, Wayne State University, Detroit, MI.

Bowles, B.L. and Miller, A.J. 1993a. Antibotulinal properties of aromatic and aliphatic aldehydes. J. Food Protect. 56: 788–794.

Bowles, B.L. and Miller, A.J. 1993b. Antibotulinal properties of aromatic and aliphatic ketones. J. Food Protect. 56: 795–800.

Brown, J.P. 1980. A review of the genetic effects of naturally occurring flavonoids, antraquinones and related compounds. Mutat. Res. 75: 243–277.

Budavari, S., O'Neil, M.J., Smith, A., and Heckelman, P.E. (Eds.) 1989. *The Merck Index, An Encyclopedia of Chemicals, Drugs, and Biologicals*, 11th ed., Merck & Co., Inc., Rahway, NJ.

Centers for Disease Control. 1974. Detection of *Clostridium botulinum* and botulinal toxin. In *Laboratory Methods in Anaerobic Bacteriology, CDC Laboratory Manual*. U.S. Dept. of Health, Education, & Welfare, Public Health Service, Center for Disease Control, pp. 41–44, Atlanta, GA.

Conner, D.E., Scott, V.N., and Bernard, D.T. 1989. Potential *Clostridium botulinum* hazards associated with extended shelf-life refrigerated foods: a review. J. Food Safety 10: 131–153.

Debevere, J.M. 1988. Effect of buffered acidulent systems on the survival of some food poisoning bacteria in medium acid media. Food Microbiol. 5: 135–139.

Gould, G.W., Stubbs, J.M., and King, W.L. 1970. Structure and composition of resistant layers in bacterial spore coats. J. Gen Microbiol. 60: 347–355.
Halvorson, H.O., Vary, J.C., and Steinberg, W. 1966. Developmental changes during the formation and breakage of the dormant state in bacteria. Ann. Rev. Microbiol. 20: 169–188.
Harborne, J.B. and Conner, J.J. 1961. Plant polyphenols 4. hydroxycinnamic acid-sugar derivatives. Biochem. J. 81: 242–250.
Hauschild, A.H.W. 1989. *Clostridium botulinum*. In *Foodborne Bacterial Pathogens*. M.P. Doyle (Ed.), p. 112–189. Marcel Dekker, Inc., New York.
Herald, P.J. and Davidson, P.M. 1983. Antibacterial activity of selected hydroxycinnamic acids. J. Food Sci. 48: 1378–1379.
Hirose, M., Fukushima, S., and Shirai, T. 1990. Stomach carcinogenicity of caffeic acid, sesamol catechol in rats and mice. Jap. J. Cancer Res. 81: 207–212.
Huhtanen, C.N. 1975. Some observations on Perigo-type inhibition on *Clostridium botulinum* in a simplified medium. J. Milk Fd. Technol. 38: 762–763.
Ismaiel, A.A. and Pierson, M.D. 1990. Inhibition of germination, outgrowth, and vegetative growth of *Clostridium botulinum* by spice oils. J. Food Protect. 53: 755–758.
Janssen, F.W., Lund, A.J., and Anderson, L.E. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science. 127: 26–27.
John, T.J. and Mukundan, P. 1979. Virus inhibition by tea, caffeine and tannic acid. Ind. J. Med. Res. 69: 542–545.
Kontoghiorghes, G.J., Jackson, M.J., and Lunec, J. 1986. *In vitro* screening of iron chelators using models of free radical damage. Free Radical Res. Commun. 2: 115–124.
Koransky, J.R., Allen, S.D., and Dowell, V.R., Jr. 1978. Use of ethanol for selective isolation of sporeforming microorganisms. Appl. Environ. Microbiol. 35: 762–765.
LaBaw, G.D. and Desrosier, N.W. 1954. The effect of synthetic plant auxins on the heat resistance of bacterial spores. Food Res. 19: 98–105.
LaBaw, G.D. and Desrosier, N.W. 1953. Antibacterial activity of edible plant extracts. Food Res. 18: 186–190.
Miller, A.J., Call, J.E., and Whiting, R.C. 1993. Comparison of organic acid salts for *Clostridium botulinum* control in an uncured turkey product. J. Food Protect. 56: 958–962.
Mountney, G. and O'Malley. 1965. Acids as poultry meat preservatives. Poultry Sci. 44: 582–586.
Nowak, H., Kujawa, K., Zadernowski, R., Roczniak, B., and KozŁowska, H. 1992. Antioxidative and bactericidal properties of phenolic compounds in rape seeds. Fett. Wissenschaft Technol. 94: 149–152.
Palumbo, S.A. and Williams, A.C. 1992. Growth *Aeromonas hydrophila* K144 as affected by organic acids. J. Food Sci. 57: 233–235.
Paster, N., Juven, B.J., and Harshemesh, H. 1988. Antimicrobial activity and inhibition of aflatoxin B_1 formation by olive plant constituents. J. Appl. Bacteriol. 64: 293–297.
Ravn, H., Andary, C., Kovacs, G., and Molgaard, P. 1989. Caffeic acid esters, as *in vitro* inhibitors of plant pathogenic bacteria and fungi. Biochem. System Ecol. 17: 175–184.
Raccach, M. 1984. The antimicrobial activity of phenolic antioxidants in foods: a review. J. Food Safety 6: 141–171.
Smith, K.T. and Dawes, J.W. 1989. The preferential inhibition of *Bacillus subtilis* spores outgrowth by chloroquine. Arch. Microbiol. 152: 251–257.
Sugiyama, H. 1990. Botulism. In *Foodborne Diseases*. Cliver, D.C. (Ed.), p. 108–125, Academic Press, San Diego, CA.
Sugiyama, H. 1951. Studies on factors affecting the heat resistance of spores of *Clostridium botulinum*. J. Bacteriol. 62: 81–96.
Toda, M., Okubo, S., Hiyoshi, R., and Shimamura, T. 1989. The bactericidal activity of tea and coffee. Lett. Appl. Microbiol. 8: 123–125.
Valle, E. 1957. Antifungal factors in potato leaves. Acta Chem. Scand. 11: 395–397.
Yamaguchi, T. and Iki, M. 1986. Inhibitory effect of coffee extract against some mutagens. Agric. Biol. Chem. 50: 2983–2988.
Ms received 10/9/93; revised 3/23/94; accepted 4/19/94.

The technical assistance of Benne Marmer, B. Shawn Eblen, and Jeffrey E. Call is greatly appreciated.

Reference to a brand or firm name does not constitute an endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned.